Metabolic Labeling of Proteins for Proteomics*

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Realization of the advantages of stable isotope labeling for proteomics has emerged gradually. However, many stable isotope label approaches rely on labeling *in vitro* using complex and sometimes expensive reagents. This review discusses strategies for labeling protein *in vivo* through metabolic incorporation of label into protein. This approach has many advantages, is particularly suited to single cells grown in culture (prokaryotic or eukaryotic), but is nonetheless subject to a number of complicating factors that must be controlled so that meaningful experiments can be conducted. Confounding issues include the metabolic lability of the amino acid precursor, incomplete labeling, and the role of protein turnover in labeling kinetics. All of these are controllable, provided that appropriate precautions are adopted. ***Molecular & Cellular Proteomics 4:•••–•••, 2005.***

The rapidly developing field of functional genomics, and proteomics in particular, have led to new approaches to the analysis of gene expression at the level of the protein (for example, see Refs. 1 and 2). The current literature is replete with new and recently reinvented approaches to the separation, analysis, and identification of proteins. The analysis of proteomes is based almost exclusively on mass spectrometric methods that make use of gentle ionization processes coupled with moderate to high resolution mass analysers to provide accurate mass determination, whether of a set of proteolytic fragments or of the intact protein. Although there are early indications of the value of protein microarrays for some aspects of quantitative proteomics (3), it is premature to suggest that arrays will supplant mass spectrometry for global proteomics. Instead, protein microarrays are likely to be focused on selective analysis of a few analytes in, for example, molecular diagnostics. The benefits of mass spectrometry include high sensitivity, universal detection (in the proteomics context), unrivalled molecular selectivity, and the opportunity for rigorous quantification.

The current generation of widely available mass spectrometers has more than adequate resolution to provide unit mass resolution across the useable mass range. At this level of performance, the possibility has arisen of developing "mass tagging" approaches for the differential analysis of protein expression. A protein or signature peptide is labeled with a tag that exists in isotopically labeled forms such that two cellular states being compared are labeled with a "light" or "heavy" variant of a tag, and the analytes are mixed before analysis. Mass spectrometric assessment of the heavy/light ratio then enables comparative expression analysis unaffected by issues such as instrument response, sample purity, and so on.

Many such mass tagging approaches use chemical labeling of complex mixtures of proteins *in vitro* after their extraction from cells. Such approaches include ICAT and its modifications (4–7), acrylamide labeling (8), ^{18}O-labeling during proteolysis (9, 10), and guanidination of lysine residues (11–13). In all of these approaches, the degree of labeling is controlled by the isotope enrichment of the labeling reagent and the completeness of the modification chemistry and is applied to proteins or protein mixtures obtained after cell disruption. However, labeling after cell breakage precludes metabolic labeling, a variant approach in which stable isotopes (usually in the form of labeled amino acids) are incorporated biosynthetically into protein *in vivo*, via the process of translation. It is timely, therefore, to address the opportunities and pitfalls associated with metabolic labeling of proteins *in vivo*.

Radioactive Versus Stable Isotopes

Labeling of proteins using radiolabeled amino acids has a long history. The specific radioactivity of radioisotopes can be so high that trace labeling can yield a readily measurable incorporation. For example, [^{35}S]methionine is commonly used because the isotope is sufficiently radioactive (~40 TBq/mmol) that even rather modest incorporation can lead to measurable accumulation of labeled material. This permits relatively short labeling time windows for incorporation of label. Furthermore, a ^{35}S label is sufficiently energetic that it is not impeded greatly by media such as polymerized acrylamide and can therefore be used successfully in autoradiography of 1DGE[1] or 2DGE. Fn1

By contrast, with the types of mass spectrometer in common usage for proteomics research, it is improbable that an incorporation of a stable isotope precursor of 1% would be detectable, because particularly in single-stage instruments the noise floor (whether chemical or instrument generated) might obscure this degree of labeling. To overcome this limitation, effective metabolic labeling would require a substantial incorporation (perhaps minimally on the order of 5–10%) for accurate quantification.

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[1] The abbreviations used are: 1DGE, one-dimensional SDS-PAGE; 2DGE, two-dimensional SDS-PAGE; RIA, relative isotope abundance; D, doubling time.

Most of the emphasis on stable isotope labeling *in vivo* has thus far been placed on comparative proteomics in which the incorporation of a metabolic label is used to identify one of the components in a pair-wise comparison (Table I). Although apparently straightforward, this approach embodies several assumptions that are not always tested rigorously. First, it is assumed that the proteins are fully labeled with the stable isotope precursor such that the heavy/light ratio can be taken to directly represent the relative amounts of the analyte in the two systems. Second, it is often assumed that the stable isotope label in the precursor amino acid is not differentially metabolized and that it does not appear in a different amino acid.

As stable isotope labeling strategies have been developed, new acronyms have been coined, such as stable isotope labeling with amino acids (SILAA) (14) and stable isotope labeling with amino acids in cell culture (SILAC) (for example, see Refs. 15–17). These acronyms refer to rather specific labeling approaches. A more general term might be "metabolic incorporation of stable isotopes," which is clearly distinguished from any stable isotope strategy whereby label is incorporated after cell lysis or preparation of the protein mixture, such as ICAT (see above).

Selection of Stable Isotope-labeled Precursor

There has been a limited set of studies in which the precursor for amino acid labeling is not an amino acid. For protein labeling, the use of metabolites such as [^{13}C]glucose can result in a variable degree of incorporation of carbon atoms into different amino acids, and therefore into protein such that comparative analyses become more complex (18). Likewise, the use of [^{15}N]H_4Cl as a precursor nitrogen source means that each peptide will incorporate a variable number of nitrogen atoms, depending on the length of the peptide and the number of nitrogen side chain-containing amino acids (19, 20). Such labeling approaches, often driven by the need to prepare proteins for nuclear magnetic resonance studies, are not ideally suited to proteomics studies. It is generally preferable to use an amino acid as the labeling precursor. The mass offset caused by the labeled amino acid can be carefully selected and even multiplexed as seen in the elegant studies of Blagoev *et al.* (21) using unlabeled arginine, $^{13}C_6$-labeled arginine (mass offset, +6 Da) and $^{13}C_6/^{15}N_4$-labeled arginine (mass offset, 10 Da) (21). Moreover, stable isotope-labeled amino acids introduce label in relatively few sites in each peptide. This obviates the complications caused by labeling a peptide with ^{15}N, whereby the combinatorial possibilities of generating partially unlabeled peptides (particularly at monoisotopic mass − 1) is evident even at 99% relative isotope abundance (RIA) of the precursor [^{15}N]H_4Cl.

In principle, any of the 20 naturally occurring amino acids could be used as a precursor for labeling proteins, but several factors conspire to diminish those choices (summarized in Table II). To a first approximation, it is reasonable to assume that all microbes and plants are competent in the synthesis of all 20 amino acids. In animal metabolism, however, a distinction is made between those amino acids that can be synthesized by the organism (the non-essential amino acids) and those that must be provided in the diet (essential amino acids). Essential amino acids are His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val. If the amino acid is non-essential, then the cell or organism is able to synthesize that amino acid from precursors that are not themselves labeled, which has the effect of reducing the relative isotope abundance of the precursor pool. It is also worth recognizing that some amino acids considered to be non-essential in intact animals are obligatory for cells in culture (possibly reflecting the roles of different cell types in biosynthesis of amino acids). For example, arginine, although synthesized in the reactions of the urea cycle, is often provided in the culture medium, presumably to avoid depletion of the arginine pool by protein synthesis, which would compromise the operation of this essential metabolic cycle. Further, some amino acids are metabolically highly labile and are rapidly deaminated or transaminated, and the carbon skeleton generated by this process is then oxidized or used in other biosynthetic pathways. This could deplete the labeled precursor or could even result in rapid recycling of label into other amino acid pools, which might then change the pattern of isotope incorporation into proteins.

In addition to diversion of the isotopic label to other metabolites, different amino acids may undergo metabolism that selectively removes some of the label from the amino acid. The α-carbon attached hydrogen/deuterium atom can be considered to be chemically stable but is metabolically labile, in that the reversible process of transamination creates the α-keto (2-oxo) acid. A good illustration of this behavior has been provided by several studies in which proteins were labeled *in vivo* with [$^2H_{10}$]leucine (Fig. 1). Although the deuterated amino acid was incorporated into protein, the masses of the products, whether analyzed as intact proteins or as tryptic fragments, were consistent with the incorporation of nine deuterium atoms for each leucine residue (22, 23). The most likely explanation was that the α-carbon deuterium atom had been lost by rapid and reversible transamination. Indeed, more recent data from our laboratory have shown that this transamination process can be tissue-specific and that liver demonstrates labeling patterns consistent with the incorporation of amino acids that have partially retained the α-carbon deuteron, whereas skeletal muscle incorporated only amino acids lacking the α-carbon deuteron (24). The incorporation of [$^2H_{10}$]leucine (and other amino acid precursors carrying deuterium at the α-carbon atom) has been used to generate amino acid composition data for use in the identification of intact proteins. Loss of a deuterium atom from the amino acid precursor is readily detected when heavy and light peptides are compared. However, when the mass comparison is made at the level of the intact protein, the decrease in mass accu-

TABLE I

FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HSF, human skin fibroblasts; YNB, yeast nitrogen base; IPTG, isopropyl β-D-thiogalactoside; trichloroacetic acid; MIDA, mass isotopomer distribution analysis; CIEF, capillary isoelectric focusing.

Organism	Label	Growth Conditions	Analytical methods	Notes
Relative quantification				
D. radiodurans B16 (mouse) cells, (50)	^{15}N-enriched medium (>98)	Celltone 98% ^{15}N-enriched yeast grown to late log phase. DMEM, 10% FBS B16 cells grown to 85% confluence (4d)	Capillary isoelectric focusing for intact proteins. Proteins denatured, cysteines labeled with iodoacetyl PEO-Biotin reagent and digested with trypsin. Cysteine peptides recovered by avidin affinity column and separated by capillary reversed-phase HPLC ESI-ion trap, ESI-FTICR	^{15}N-enriched medium is cheap, but label is incorporated into other macromolecules and the mass difference between heavy and light forms of the same peptide is variable because it is sequence-dependent. ^{15}N-labeled peptides display minor isotope-dependent chromatography shifts relative to their ^{14}N-labeled counterparts
S. cerevisiae Methionine auxotroph (29)	[$^{2}H_3$]methionine (99.9) [$^{2}H_2$]tyrosine (99.9) [$^{2}H_3$]serine (99.9)	Batch culture, minimal medium, containing 0, 50, or 100% labeled amino acid. Cells harvested in log phase.	1DGE, electroelution from gel slices, TCA precipitation. 2DGE, in gel digestion. Tryptic peptides analyzed by MALDI-TOF.	Only 2–3-Da shift between heavy and light peptides leading to isotopomer overlap.
D. radiodurans, E. coli, S. cerevisiae, Mammalian cells (51)	Isotopically depleted (~99.95% ^{12}C, ~99.99% ^{14}N and >99.995% ^{1}H) medium $_{15}$N-enriched medium (>98)	Cells grown in ^{15}N-enriched medium	CIEF, tryptic peptides LC-FTICR	Nitrogen atom composition aids protein identification. Identify peptides to act as accurate mass tags for a given protein within a proteome. The mass alone of a single peptide can be used as a biomarker for unambiguous identification.
S. cerevisiae Leucine auxotroph (52)	L-[$^{2}H_{10}$]leucine (99.4)	Cells grown in synthetic complete medium at 30 °C and harvested at stationary phase	2DGE Tryptic peptides analyzed by MALDI-TOF	Observed that in $^{2}H_{10}$-labeled peptides, one deuteron is exchanged for hydrogen, but presumed this to occur in the MALDI source.
Mouse C2C12 myoblast cells, NIH 3T3 fibroblasts (17)	L-[$^{2}H_3$]leucine (99)	Eagle's minimal medium plus serum, six cell divisions	1DGE. Tryptic peptides analyzed by MALDI-TOF, ESI-QTOF	Dialysis of serum reduces input of unlabeled amino acid. Web based MS-Isotope used to facilitate analysis.
D. radiodurans (19)	^{15}N-enriched medium (>98)	Cells grown in ^{15}N-enriched medium. Hydrogen peroxide stress.	Denatured, tryptic peptides, gradient ion exchange chromatography. LC-FTICR MS/MS	Expression changes in response to oxidative stress. Further development of the use of accurate mass tags.
●● (53)	^{15}N-enriched medium	Recombinant proteins PTP and HtrA labeled with ^{15}N or ^{14}N	Proteins mixed in a variety of known ratios. Tryptic peptides. MALDI-TOF, LC MS.	Use inverse labeling of tryptic peptides to confirm very large apparent expression changes.
S. cerevisiae Human skin fibroblasts (54)	L-[$^{2}H_3$]leucine (98) L-[$^{2}H_3$]serine (98) L-[$^{2}H_2$]tyrosine (98)	*S. cerevisiae* grown in chelated synthetic defined medium. Cells harvested at mid-late log phase.	2DGE analysis of yeast. HSF cells by 1DGE. Tryptic peptides analyzed by MALDI-ToF and LC ESI/MS (ion trap)	Effect on *S. cerevisiae* protein expression of zinc depletion in Zap1 mutant. Effect of irradiation on protein expression in HSF cells.
C. elegans D. melanogaster (20)	^{15}N-enriched medium (98)	*E. coli* and *S. cerevisiae* grown on ^{15}N-enriched medium and fed to *C. elegans* and *D. melanogaster*, respectively	2DGE, tryptic peptides, MALDI-ToF Nano-LC Q-ToF, LC-MS/MS	Whole organism metabolic labeling achieved 94–95% labeling. Quantified differences in protein profile between WT and glp4 mutant of *Caenorhabditis elegans.*
NIH 3T3 fibroblasts (36)	L-[$^{13}C_6$]arginine	Eagle's medium with all amino acids except arginine. Supplemented with glutamine and FBS. Although arginine is not an essential amino acid, it is essential in cell culture.	1DGE, tryptic peptides, LC-MS, LC-MS/MS	Use of ^{13}C as isotope ensures co-elution of heavy and light peptides during LC. Peptides containing [^{13}C]proline were found, presumably arising from arginine catabolism. Excellent quantification was achieved with S.D. of 2–3%.
D3 embryonic cells (55)	Seven ^{15}N-labeled amino acids (>99): L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glycine, L-serine and L-glutamine	Modified Eagle's medium with 15% FCS. 12 days growth, 4 passages.	2DGE, tryptic peptides, MALDI-TOF	Determined error on quantification of various ratios of heavy and light protein lysate, 4–18% error in quantification. The ^{15}N label could be transferred to other amino acids by transamination.

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TABLE I—*continued*

Organism	Label	Growth Conditions	Analytical methods	Notes
Human HeLa cells (48)	L-[$^{13}C_6$]arginine (99) L-[$^{2}H_3$]leucine	Arginine or leucine deficient DMEM with 10% FBS, 5 cell doublings. Cells grown in labeled medium stimulated with EGF.	50/50 labeled with unlabeled cells, immunoprecipitated. 1DGE, trypsin digestion and LC-MS/MS. Peptides (non-phosphorylated and phosphorylated forms) immobilized to beads were used to affinity purify interacting proteins from labeled and unlabeled lysates, then mixed 50:50 before 1DGE, trypsin digestion and analysis by LC-MS/MS.	All peptides are paired; those that change with EGF stimulation are unequal in intensity. Peptides derived from proteins specifically binding to phosphorylated peptide seen only as heavier form. Those derived from proteins bound through non-specific interactions or derived from proteins bound equally to phosphorylated and non-phosphorylated forms, showed no difference in intensity.
Human prostate carcinoma cell lines PC3 M and PC3M-LN4, showing low and high metastatic potential respectively. (16)	L-[$^{13}C_6$]lysine (98)	L-lysine depleted RPMI 1640 medium. Cells grown to 60–80% confluence.	Microsomal fractions from two cell types were mixed 1:1 (PC3M-LN4 cells labeled), boiled in SDS and separated on 1DGE. Gel slices were digested, tryptic peptides extracted and analyzed by LC-MS with automated selection for MS/MS. Quantification used MSQuant software.	Expression of 60 proteins elevated in highly metastatic cells.
Plasmodium falciparum lines HB3 and Dd2 (33)	L-[$^{13}C_6{}^{15}N_1$]isoleucine (>98)	O^+ erythrocytes in RPMI without isoleucine. Synchronous growth by sorbitol treatment. Asynchronous cultures for effect of drugs.	Parasites released from erythrocytes, analyzed by 2DGE. Tryptic peptides, MALDI-TOF and LC-MS/ MS/MS.	Study of proteome changes during the malarial parasite cell cycle and identification of changes induced by antimalarial drugs.
Rattus norvegicus (38)	^{15}N-labeled (>99) algal cells	Rats were fed for 44 days on a protein-free rodent diet with unlabeled or ^{15}N-labeled algal cells.	Tissue lysates were generated from rats given cycloheximide 4 h before sacrifice. 1DGE, Proteinase K digestion, MudPIT, LC-MS, MS/MS	The ^{15}N-labeled tissue was used exclusively as an internal standard for tissue lysates derived from rats treated with cycloheximide. 127 proteins with altered levels were identified.
HeLa cells (21)	L-[$^{13}C_6$][$^{14}N_4$]-arginine L-[$^{13}C_6$][$^{15}N_4$]-arginine	Amino acid free DMEM with labeled or unlabeled arginine. Treated with epidermal growth factor (EGF) for 0, 1 or 10 min.	Mixed lysates were immunoprecipitated with anti-phosphotyrosine antibody, separated on Bis-tricine gels and tryptic peptides from gel slices analyzed by LC-MS.	Use of arginine of three different masses allowed simultaneous quantification of three cellular states; 81 signaling proteins were identified.
HeLa cells (56)	L-[$^{13}C_6$][$^{14}N_4$]-arginine L-[$^{13}C_6$][$^{15}N_4$]-arginine	Treatment with RNA polymerase I, II, or proteasome inhibitor, 5–9 separate time-points.	Nucleoli isolated using density gradients. 1DGE, tryptic or endopeptidase Lys-C peptides, LC-MS/MS on an ion trap-Fourier transform MS.	Characterized the flux of 489 endogenous nucleolar proteins in response to three metabolic inhibitors.
Identification				
E. coli Leucine auxotroph (39)	L-[$^{2}H_{10}$]leucine	Minimal medium	CIEF, FTICR MS	Intact proteins, assumed heavy leucine incorporated as [$^{2}H_{10}$]leucine rather than [$^{2}H_9$]leucine.
E. coli Glycine or methionine auxotroph (57)	L-[$^{2}H_3$]methionine (99.9) [$^{2}H_2$]glycine (99.9)	Minimal medium with 50% labeled amino acid, or LB (control), expression of His-tagged protein UBL1 (ubiquitin-like protein) induced by IPTG.	His-tagged protein purified. Tryptic peptides, MALDI-ToF, MALDI-ToF PSD	2–3-Da mass difference results in overlapping spectra for heavy and light peptides and growth in 50% label further complicates spectra. Partial composition data aids protein identification.
S. cerevisiae Lysine auxotroph (35)	[$^{13}C_6$]lysine (98)	Synthetic medium, stationary phase	Denatured proteins, endopeptidase Lys-C peptides, capillary LC, ESI-FTICR	Automation via data-dependent multiplexed MS/MS for labeled peptide pair selection.
E. coli HSF (human) cells (58)	L-[$^{2}H_4$]lysine (96–98)	Induced expression of His-tagged protein in *E. coli* in minimal medium with 50% labeled lysine. HSF cells grown to 80% confluence in Invitrogen MEM medium with 50% labeled lysine.	Protein purified before analysis. Endopeptidase Lys-C or tryptic peptides. MALDI-ToF, LC-MS/MS ESI-ion trap,	Analysis of recombinant proteins. Endopeptidase Lys-C digestion to generate C-terminally labeled peptides and facilitate analysis of MS/MS spectra.

TABLE I—*continued*

Organism	Label	Growth Conditions	Analytical methods	Notes
E. coli Leucine auxotroph *S. cerevisiae* Leucine auxotroph (30)	L-[$^2H_{10}$]leucine L-[$^2H_{10}$]isoleucine L-[$^2H_8$]phenylalanine L-[$^{13}C_6$]arginine L-[$^{13}C_6$]histidine L-[$^{13}C_6$]lysine	Minimal medium for *E. coli.* SC medium for *S. cerevisiae.*	CIEF, FTICR MS	Intact proteins, assumed heavy leucine and isoleucine incorporated as [$^2H_{10}$] rather than [$^2H_9$]amino acid. Knowing the composition of 3–4 of the amino acids in a peptide is sufficient for unique identification even in complex proteomes.
S. cerevisiae (22)	D,L-[$^2H_{10}$]leucine (98.5)	Continuous culture in glucose limited medium at growth rate of 0.1/h	2DGE, Tryptic peptides MALDI-ToF	Use of peptide leucine content to reduce search space for protein identification.
HSF (31)	L-[$^2H_3$]leucine L-[$^2H_4$]lysine L-[$^2H_3$]methionine L-[$^2H_3$]serine L-[$^2H_2$]tyrosine	Cell culture medium, 10% FBS Cells grown to 80% confluence in 50% label	1DGE, Tryptic peptides MALDI-ToF, LCQ MS/MS	Identification of protein from mass of single peptide and partial composition. Mass differences of 2–3-Da between heavy and light peptides are insufficient for good resolution in MS. Growth in 50% label also complicates the interpretation of spectra.
Turnover				
Human, rat (59)	Any label that is incorporated multiple times into a polymer /large molecule (protein, nucleic acid, porphyrins, fatty acid, cholesterol, including glucose, amino acids, [2-^{13}C]acetate for biosynthesis of fatty acids and cholesterol.	Whole animal experiments, humans, rats (fasted or fed)	MS analysis of polymer to measure mass isotopomer distribution. A comparison is made of the statistical distributions predicted from the binomial or multinomial expansion to the pattern of excess isotopomer frequencies observed in the polymer, the enrichment of the biosynthetic precursor subunits for newly synthesized polymers is calculated.	MIDA, a technique for measuring biosynthesis and turnover of polymers without knowing the precursor pool atom percent excess. By combining fractional synthesis values with rate constants of decay, absolute endogenous synthesis rates can be calculated.
Rainbow trout, *Oncorhynchus mykiss* (60)	^{15}N-labeled yeast	Trout fed a measured weight of labeled food for 6 h, then transferred to a new tank and water samples taken for 48 h.	Ammonia was distilled into HCl after water samples were made alkaline with NaOH. ^{15}N enrichment was determined using an ANCA Roboprep-CN linked to a tracer mass isotope ratio mass spectrometer.	Developed a non-invasive method that uses ^{15}N in orally administered protein to measure protein synthesis rates in fish.
Male Sprague-Dawley rats (61)	L-[$^2H_3$]leucine (99)	Infusion via jugular vein for 24 h, ± feeding.	Sampled rat muscle, serum, abdominal and cardiac tissue. Isolated creatine kinase, CK (size exclusion HPLC), and myosin heavy chain, MHC from rat muscle. Purification of L_5 peptide by HPLC. Analysis by ESI/MS. MHC and CK acid hydrolyzed and enrichment of [$^2H_3$]leucine measured by GC/MS.	MIDA used to measure isotopic enrichment of the true biosynthetic precursor for proteins, tRNA-amino acids. Although enrichment of mass isotopomers in the CK peptide was measurable, it was very low (indicating a slow turnover rate of 3–10 days).
S. cerevisiae Leucine auxotroph, diploid. (23)	D,L-[$^2H_{10}$]leucine (98.5)	Continuous culture in glucose limited medium at growth rate of 0.1/h.	2DGE, tryptic peptides MALDI-TOF	Chemostat growth ensured absence of fluctuations in medium occurring during batch growth. Seven generations ensured 99.9% labeling of yeast proteins.
Male Swiss mice (62)	L-3-methyl-[$^2H_3$]histidine L-[$^2H_5$]phenylalanine L-[$^2H_2$]tyrosine	Continuous infusion of labeled amino acids.	Mouse serum collected, LC-MS	Whole animal study. Breakdown of actomyosin leads to the release of 3-methyl histidine, which cannot be reutilized and is excreted in the urine. Used to quantify myofibrillar protein breakdown in different conditions.
E. coli (18)	[$^{13}C_6$]glucose	Minimal medium, mid log phase growth, 30 min with added label.	1DGE, tryptic peptides from gel slices separated by HPLC, spots of eluant collected on MALDI target, MALDI TOF-TOF for analysis. The isotopomer envelope for the heavy peptide is spread over a wide range of masses, reflecting the metabolic promiscuity of glucose as label.	Measuring synthesis/degradation ratio, ie how rapidly a newly synthesized protein builds up as a function of the rate of the degradation of that protein relative to other cellular proteins. This method requires that no peptides co-elute, to avoid overlapping spectra, and the identification of an accurate method for quantification of the broad heavy peak.

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TABLE I—*continued*

Organism	Label	Growth Conditions	Analytical methods	Notes
Modification				
S. cerevisiae	^{15}N-enriched medium (>96)	Rich medium, harvested mid log phase. Galactose induced synthesis of Ste20 protein.	HPLC (C4) of intact proteins 1DGE Tryptic peptides analyzed by MALDI-TOF. LC-ESI-MS/ MS	Copper staining to visualize bands. Identified four phosphorylation sites of the Ste20 protein.
S. cerevisiae Human skin fibroblasts (HSF) (32)	L-[$^{2}H_3$]methionine (99.9) D,L-[$^{2}H_3$]serine (99.9)	*S. cerevisiae* grown to late log phase in defined medium with 0, 50 or 100% label. HSF cells 12h in labeled medium.	1DGE. Tryptic peptides PE-Voyager DE-STR Delayed extraction	GST fusion protein with kinase A site. Methionine oxidation and protein phosphorylation.
Human embryonic kidney 293T cells (32)	L-[$^{13}C_6$]lysine L-[$^{13}C_6$]arginine	DMEM lacking lysine and arginine	Calyculin A treatment of cells. Immunoprecipitation to enrich FRIGG protein. 1DGE, tryptic digestion, LC-MS/ MS. Analysis of peptide pairs of a 50:50 mixture of unlabeled untreated and labeled treated cells identified peptide pairs.	Peptides with differing ion intensity (as much as 1:9 labeled to unlabeled) resulted from an increase in the proportion of phosphorylated peptide in the calyculin A-treated sample. Sites of phosphorylation are readily identified by unequal intensities of peptide pairs.
3T3-L1 and HeLa cells (44)	L-[$^{13}C_9$]tyrosine	DMEM without tyrosine	3T3-L1 cells were differentiated into adipocytes and stimulated with insulin. Immunoprecipitation of phosphotyrosine proteins. 1DGE, LC-MS of tryptic peptides.	6 days of cell culture resulted in complete labeling of cells. Only tyrosine containing tryptic peptides appear as doublets. Phosphopeptides identified.
S. cerevisiae (63)	L-[$^{13}C_6$]lysine L-[$^{13}C_6$]arginine	YNB medium without arginine and lysine	Lysates analyzed by 1DGE, gel slices, tryptic digestion or lysate treated with endoproteinase Lys-C and cation exchange chromatography. Phosphoproteins enriched by IMAC. LC-MS, MS/MS.	Identified changes in the phosphopeptide proteome after treatment with yeast mating pheromone.
Specific applications				
Mouse (1)	L-[$^{13}C_2$]glycine	Added to the diet	RNA-bound purine nucleosides extracted and enzymatically degraded to glycine, analyzed by GC-MS.	Identified two pools of free nucleotides; one acts as a precursor for RNA, the other as a pool of adenosine triphosphate for cellular energetics.
Mouse myeloma cells (64)	L-[$^{2}H_{10}$]leucine (98)	Cell culture in defined medium	IgG in culture supernatant, denatured, endopeptidase Lys-C or staphylococcal V8 protease peptides analyzed by HPLC MALDI-TOF FAB MS/MS, ESI MS/MS	Discrimination of leucine from isoleucine. Mapping of peptides to a given protein from mass and leucine content.
E. coli (28)	[$^{2}H_3$]methionine [$^{2}H_2$]glycine [$^{2}H_2$]tyrosine [$^{2}H_4$]lysine [$^{2}H_3$]leucine	Minimal medium, supplemented with amino acids. IPTG induction of His-tagged human ubiquitin conjugating enzyme 9.	Mixed labeled and unlabeled purified protein and refolded. Pronase treated in D_2O. LC-MS, MS/MS, ESI-ion trap.	Identification of pepsin-derived peptides in hydrogen exchange studies.
E. coli (65)	^{15}N enriched medium	Recombinant interleukin 6 expressed in *E. coli* grown in ^{15}N-enriched medium and ^{14}N-labeled medium and purified.	Heavy and light IL6 denatured, mixed 50:50 and refolded. Bifunctional cross-linking, 1D SDS-PAGE, tryptic peptides of dimer band. LC-MS/MS.	Identify inter- and intramonomer interactions.
HeLa cells (15)	L-[$^{13}C_6$]arginine	Eagle's minimal medium plus serum, six cell divisions	Affinity purification of SH2-tagged fusion protein and complexed proteins. Tryptic peptides, LC-MS/MS.	Identified EGF receptor interacting proteins from cells with or without EGF stimulation.

TABLE II
Representative stable isotope amino acid precursors for metabolic labeling studies

Amino Acid	Accessible Atoms, Representative Stable Isotope-Labeled Variants	Notes
Alanine C:3, N:1, H:(3 + 1)[a]	U-[$^{13}C_3$]Ala 3-[^{13}C]Ala 1-[^{13}C]Ala, 2,3,3,3-[$^{2}H_4$]Ala	Transamination to pyruvate, and thence to lactate, which makes the alanine pool metabolically very mobile.
Arginine C:6, N:4, H:(6 + 1)	U-[$^{13}C_6$]Arg Guanidino-[$^{15}N_2$]Arg 2,3,3,4,4,5,5-[$^{2}H_7$]Arg	Susceptible to the action of arginase to form urea and ornithine, which might result in loss of guanidine label. Ornithine converted to glutamate semialdehyde, which is cyclized to pyrroline 5 carboxylate, a precursor of proline. Appearance of ^{13}C label through metabolism of Arg to Pro seen experimentally (36). Convenient in analysis of tryptic peptides (and all endopeptidase ArgC-generated peptides), as all Arg-terminated peptides will contain a single stable isotope label. Helps in *y* ion assignment of MS/MS spectra for peptide sequencing.
Aspartate, asparagine glutamate, glutamine,	Many variants	The acidic amino acid and their amides are so actively metabolized and play such a central role in amino acid deamination that label might be very rapidly lost or redistributed. If these amino acids are to be used it would be important to track the fate of the label in preliminary experiments.
Glycine C:2, N:1, H:(2)	U-[$^{13}C_2$]Gly 2,2-[$^{2}H_4$]Gly	Maximal 2 amu separation of heavy and light causes difficulty in separation of natural [^{13}C]isotopomer envelopes.
Histidine C:6, N:3, H:(4 + 1)	U-[$^{13}C_6$]His U-[$^{15}N_3$]His 1,2,2-[$^{2}H_3$]His	Essential amino acid. Histidine is degraded to glutamate.
Isoleucine C:6, N:1, H:(9 + 1)	U-[$^{13}C_6$]Ile	Essential amino acid. Allows differentiation between isoleucine and leucine.
Leucine C:6, N:1, H:(9 + 1)	U-[$^{13}C_6$]Leu 2,3,3,3,5,5,5,5′,5′,5′-[$^{2}H_{10}$]Leu	Essential amino acid. Rapidly transaminated, removing the α-carbon deuteron (23). Allows differentiation between Leu and Ile residues.
Lysine C:6, N:2, H: (8 + 1)	U-[$^{13}C_6$]Lys 4,4,5,5 [$^{2}H_4$]Lys 3,3,4,4,5,5,6,6 [$^{2}H_8$]Lys	Essential amino acid. Degraded to acetyl-CoA and could in principle recycle label to other amino acids Convenient in analysis of tryptic peptides (and all LysC-generated peptides), as all Lys-terminated peptides will contain a single stable isotope label. Helps in *y* ion assignment of MS/MS spectra for peptide sequencing. Readily available in 4-atomic mass unit variants.
Methionine C:5, N:1, H:(7 + 1)	U-[$^{13}C_5$]Met methyl-[$^{2}H_3$]Met	The *S*-methyl group can be a highly metabolically active methyl donor and label specific to this position could be very labile. Methionine is a precursor of cysteine.
Phenylalanine C:9, N:1, H:(7 + 1)	Ring-[$^{13}C_6$]Phe U-[$^{13}C_9$]-Phe Ring (2,3,4,5,6)-[$^{2}H_5$]Phe α,β,β,2,3,4,5,6-[$^{2}H_7$]Phe	Essential amino acid. Phenylalanine is readily converted to tyrosine by phenylalanine hydroxylase.
Serine C:3, N:1, H:(3 + 1)	U-[$^{13}C_3$]-Ser 2,3,3-[$^{2}H_3$]-Ser	Degraded to pyruvate. Synthesized from glycolytic pathway precursor. Maximal 3-atomic mass unit separation of heavy and light causes difficulty in separation of natural [^{13}C]isotopomer envelopes. Marks serine-containing peptides to aid studies on serine phosphorylation.
Tryptophan C:11, N:2, H:(7 + 1)	U-[$^{13}C_{11}$]-Trp	Given the scarcity of tryptophan in proteomes, there seems little justification for use of this amino acid.
Tyrosine C:9, N:1, H:(7 + 1)	Ring-[$^{13}C_6$]-Tyr U-[$^{13}C_9$]-Tyr β,β-[$^{2}H_2$]-Tyr ring (2,3,5,6)-[$^{2}H_4$]-Tyr	Degraded to acetyl-CoA. Can be used to label tyrosine-containing peptides to aid studies on tyrosine phosphorylation.
Valine C:5, N:1, H:(7 + 1)	U-[$^{13}C_5$]-Val [^{15}N], U-[$^{13}C_5$]-Val [$^{2}H_8$]-Val	Essential amino acid. Readily transaminated, removing the α-carbon deuteron.

[a] The designation H(n+1) refers to the number of hydrogen atoms (n) other than those associated with the carboxyl and amino groups, in addition to the α-carbon hydrogen (referred to by "+1").

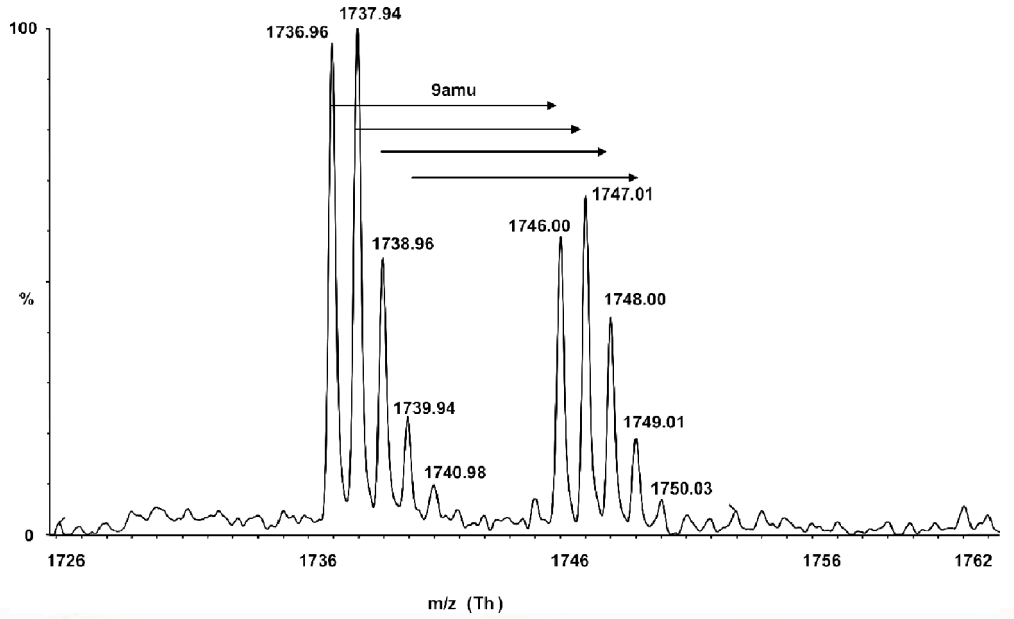

FIG. 1. **Mass offset as a result of incorporation of a stable isotope-labeled amino acid.** In this example, proteins from *S. cerevisiae* were fully labeled with [$^2H_{10}$]leucine at a relative isotope abundance of unity. The cells were mixed with an approximately equal number of unlabeled cells, grown under identical conditions, and proteins were purified by 2DGE. Peptide mass fingerprinting from a single spot showed the expected 1:1 mixture of heavy and light peptides (a single peptide over a narrow range of *m/z* values is shown here). However, the separation between the two isotopomers was 9 atomic mass units, not the 10 atomic mass units expected from the precursor labeling pattern. This is because the α-carbon deuteron, although chemically stable, is metabolically labile and is lost in the reversible process of transamination. A similar loss of label would be expected with ^{15}N-labeled amino acids because the nitrogen label in the α amino group would also be vulnerable to transamination.

racy can obscure this loss, causing miscalculation of the number of leucine residues in the protein and possibly misidentification. Therefore, when studying intact proteins, either the fate of the α-carbon in a given tissue must be determined or precursors deuterium-labeled at the α-carbon should be avoided. Of course, any loss of label should be immediately apparent in mass spectrometric analysis of peptides derived from the protein. This need not be a specific protein in that any protein synthesized in the same tissue and subcellular compartment will report on the isotope labeling pattern of the precursor.

Amino acids that are uniformly labeled with ^{15}N will incorporate stable isotope into the α-amino group. Again, it is worth remembering that this amino group is transferred to another amino acid (often 2-oxo glutarate, yielding labeled glutamate) and that this has the potential not only to reduce the mass offset, but also under extreme circumstances might deliver label via another amino acid.

The amino acids used most commonly for stable isotope studies are those that are least likely to contribute isotopically labeled atoms to general metabolic pools. Otherwise, this loss not only reduces the amount of label available for incorporation but also increases the chance of isotope appearing in other protein precursors. For example, labeled glutamate would be rapidly transaminated to 2-oxoglutarate and enter the tricarboxylic acid cycle. From there, labeled carbon atoms would be able to enter aspartate via oxaloacetate. Likewise, labeled alanine would generate the corresponding keto acid, pyruvate, which could be converted to lactate, or to acetyl CoA, leading in the latter instance to a range of metabolic products. For such reasons, most studies have used leucine, followed by lysine, arginine, and to a lesser extent serine, glycine, histidine, methionine, valine, and tyrosine. The isotope used in most of these early studies was predominantly ^2H, although more recent studies have switched to ^{13}C-labeled amino acids. This is almost certainly because of the propensity of ^2H-labeled peptides to migrate differently from ^1H counterparts under chromatographic conditions, particularly on reversed-phase HPLC (25–27).

A further consideration in the selection of the most suitable amino acid is the abundance of that amino acid in any proteome. Amino acids such as leucine and serine are the most abundant at around 10% of all amino acids (commensurate with each having six codons), whereas at the other extreme, tryptophan and cysteine are relatively rare (around 1% of amino acids in a proteome). Fig. 2 maps the correlation of F2
amino acid abundances among four proteomes; those of the yeast *Saccharomyces cerevisiae*, the chicken *Gallus gallus*, the bacterium *Salmonella typhimurium*, and the malaria parasite *Plasmodium falciparum*. Although the correlations are strong, and the overall ranking of abundance is similar, the differences between proteomes are sufficient to advocate for a quick analysis of amino acid frequencies. In particular, the genome of *P. falciparum* has a high A+T content (76% AT in

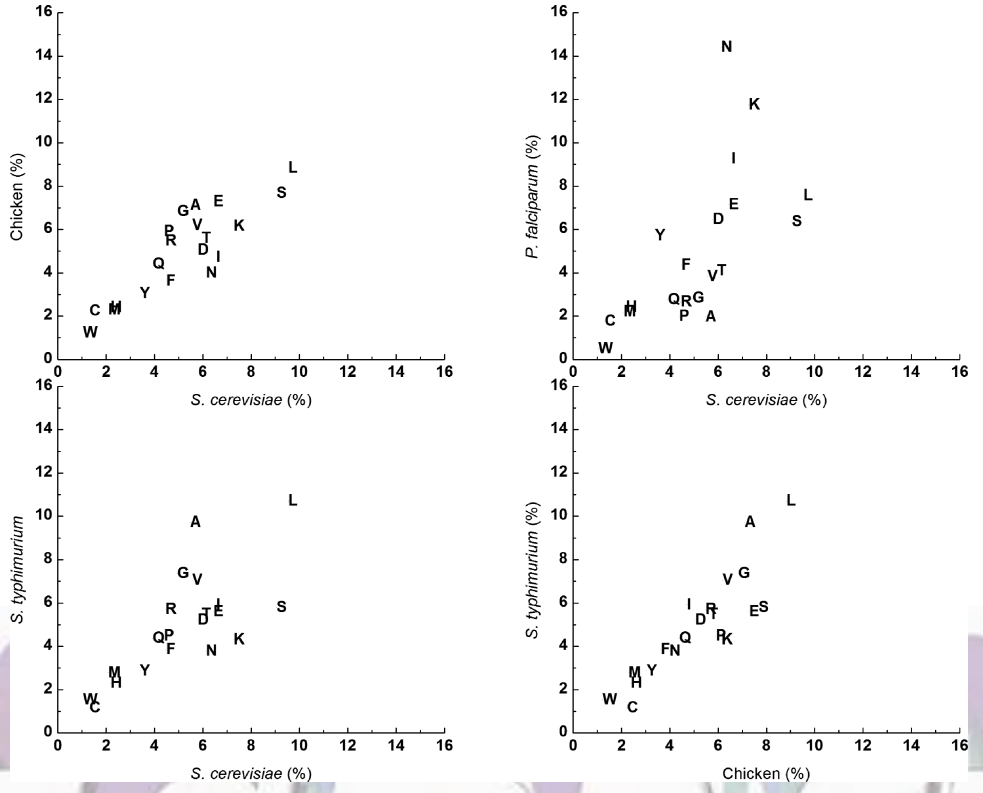

FIG. 2. **The amino acid abundance in different proteomes.** Four genomes are compared in a subset of four pair-wise correlations, expressing the abundance of each amino acid (labeled according to the one-letter code) in the proteome of one species relative to another.

coding regions, 81% overall), which has a marked effect on the amino acid distribution within the proteome (www.plasmodb.org). Compared with the yeast proteome, lysine and asparagine residues are markedly elevated (codons are AAA, AAG and AAT, AAC, respectively) and arginine residues (codons CGT, CGC, CGA, CGG) are notably depressed (Fig. 2).

A final factor, of course, relates to the availability, commercial or otherwise, of specific stable isotope amino acid variants. Some of the variants that have been used are listed in Table II, although new variants are becoming available and will not be included here. The smaller amino acids have fewer labeling centers that are chemically or metabolically accessible or stable, and the mass difference between the stable isotope variant and the unlabeled amino acid will be smaller. This becomes an important consideration when the quantification of the heavy and light variants becomes necessary. Because the natural isotope distribution of the other amino acids remains unchanged, the heavy and light peaks are complex, and with a small mass difference can overlap quite considerably. Whereas it is possible to deconvolve such overlapping distributions and quantify the heavy and light peaks, this is a complex and iterative process, requires high quality data, and is tedious. There is much to be gained by use of an amino acid, where the heavy/light mass difference is at least 4 Da.

Separation Methods

A number of different separation methods have been used to resolve complex mixtures of intact proteins or peptides. These separation methods are variously sensitive or insensitive to the presence of stable isotope-labeled amino acids. For the separation of intact proteins, 1DGE or 2DGE, capillary isoelectric focusing, ion exchange chromatography, and HPLC (C4) have been used. Only HPLC has shown differential elution of heavy and light forms of the same protein if a ^{2}H or ^{15}N label is incorporated, and this is more pronounced if proteolytic peptides are separated by HPLC (C18) (25–27). In general, to facilitate downstream analysis and automation, separation using liquid chromatography is improved by use of ^{13}C-labeled amino acid precursors. Though more expensive, the initial cost is arguably offset by the speed and facility of data analysis.

Uses of Metabolic Labeling with Stable Isotopes

There are three major categories of experiment that have used metabolic labeling with stable isotopes in proteomics. First, comparative studies emphasize assessment of the relative intensities of a heavy/light pair of analytes (usually limit peptides) to compare expression levels of different proteins. Second, incorporation of a labeled amino acid can be used to "count" the number of amino acids in specific peptides. We

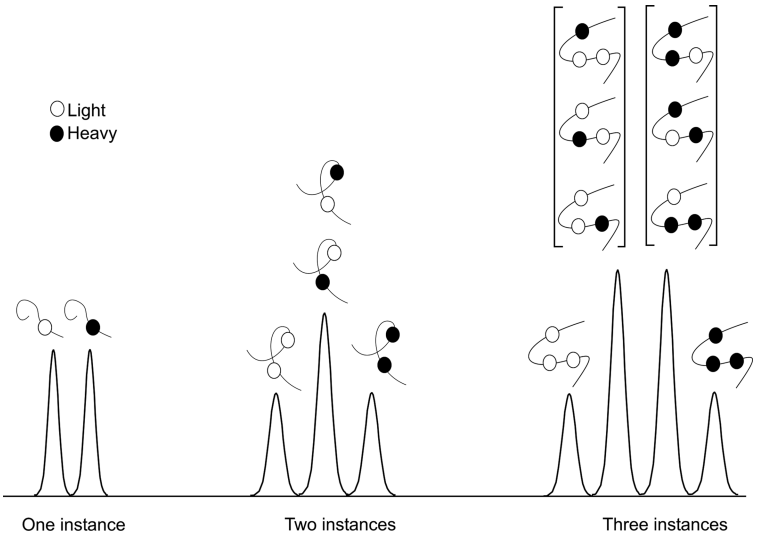

FIG. 3. **Isotopomer labeling patterns in peptides after metabolic incorporation of amino acids.** In this example, it is assumed that the precursor RIA is 0.5 and therefore that the abundances of the heavy and light amino acids are equal. For a peptide containing one instance of the labeled amino acid, the heavy and light peptides are present in equal amounts. However, for peptides containing higher numbers of the amino acid, the pattern becomes more complex according to a binomial expansion. If the RIA is 0.5, the isotopomer distributions are symmetrical, but any deviation from this value will also skew the distribution of the isotopomer peaks.

(22), and others (28–32), have shown that the reduction in search space with such limited composition information is substantial (see below). This is optimal when the mass offset between the light and heavy pair is clearly differentiated from the natural ^{13}C isoptopomers and when the precursor RIA is close to unity (see below). Finally, although most studies use fully labeled proteins, partial labeling through the use of shorter exposure times to precursor means that the extent of incorporation of label becomes a function of the rate of synthesis of specific proteins; it follows that the extent of incorporation can then be used to determine the rate of synthesis of the protein that donated the corresponding peptide (24).

Although most studies have focused on peptides derived from isolated proteins or from complex mixtures, some studies have emphasized mass measurement of intact proteins. In this instance, the mass offset between the light and heavy variants can be used to determine the abundance of a particular amino acid in a protein (30).

The Necessity for Complete Labeling of Precursor Pools

The RIA of most commercially produced stable isotope-labeled amino acids exceeds 97%, and to a first approximation can be considered as homogeneous in terms of the stable isotope profile (although natural ^{13}C distributions will usually be unchanged in ^{2}H-labeled amino acids). High precision analysis might require resolution of the heavy and light variants caused by incorporation of an incompletely labeled precursor, but this is rare. Most studies employ the stable isotope as the sole label because this simplifies the incorporation kinetics and profile of labeled peptides. If the precursor RIA is ~1.0 and proteins have been almost completely replaced by protein turnover, then there can only be fully light or fully heavy peptides, irrespective of the number of amino acids in the peptide. However, if the precursor RIA is less than 1, the mass isotopomer distribution becomes more complex.

Consider a simple experiment in which a precursor amino acid RIA has been set at 0.5 by appropriate mixing of labeled and unlabeled forms. As the amino acid is incorporated into protein one residue at a time, it has a 50/50 chance of being heavy or light, creating a complex mixture of protein molecules that differ both in the numbers of heavy amino acids and in their positions. Although analysis of the intact protein is problematic because of the distribution of signal over large numbers of variants of the protein of subtly different mass, it is feasible to explore these distributions at the level of the tryptic peptides. For example, a peptide with two instances of a particular amino acid will exit in four positional variants: HH, HL, LH, and LL, the middle two of which have the same mass; there are therefore three mass variants. If the precursor RIA is 0.5, the three mass peaks will exist in the ratio 1:2:1. For a peptide containing three instances of the amino acid, the distribution (H_3, H_2L, HL_2, L_3) will follow the binomial expansion and yield an abundance ratio of 1:3:3:1 (Fig. 3). F3

In single-cell systems, such as bacteria, yeast, animal, or plant cells maintained in culture, there seems to be little justification for complication of the isotope distribution patterns in this way irrespective of the experiment. Appropriate design of growth media and in some cases use of mutant strains such as amino acid auxotrophs (strains that have lost the ability to synthesize a particular amino and that therefore have an obligatory requirement for that amino acid in the external milieu) allow the experimenter to set the precursor RIA to near unity. It is useful when first evaluating a labeling strategy to analyze a sample in which equal amounts of labeled and unlabeled proteins/peptides are present. This sample is best generated by mixing equal quantities of unlabeled and fully labeled sample rather than by labeling in the presence of labeled precursor at an RIA of 0.5.

Intact multicellular organisms (and particularly animals) present additional complexities and require novel solutions. It is possible, for example, to obtain stable isotope-labeled nematode worms by feeding them on uniformly labeled bac-

teria (20). Proteins in the malaria parasite *P. falciparum* can be labeled by growing the parasites in medium containing human erythrocytes and labeled isoleucine. Human hemoglobin contains no isoleucine residues, and *P. falciparum* cannot synthesize this amino acid *de novo*. Therefore, isoleucine must be included in the medium, and if labeled with stable isotopes can be used for specific, high abundance labeling of the proteome. This elegant study also proved that the labeling was efficient, that the degree of incorporation attained nearly 100%, and that by implication the precursor RIA was close to unity (33).

However, experiments in intact organisms (and particularly in animals) usually cannot be designed so that the precursor RIA is unity. First, this would probably entail a synthetic diet with attendant issues of palatability. Second, any pre-existing protein in the body of the animal before the experiment began would be catabolized intracellularly and therefore contribute unlabeled amino acids to the labeled precursor pool, diluting that pool and diminishing the value of RIA. In such experimental systems, it is inevitable that the precursor RIA will be less than unity. Therefore, the isotope incorporation profiles become more complex, and more rigorous analysis of the mass isotopomers is required (24, 34, 38).

Stable Isotope-labeled Amino Acids for Comparative Proteomics

Comparative proteomics is the most common application of stable isotope metabolic labeling, and most studies have used single cell organisms or mammalian cells in culture (Table I). Proteins are labeled *in vivo* by introduction of label in the growth medium. Labeled and unlabeled cells are then mixed, and subsequent proteome analysis compares the intensity of the light and heavy variants to obtain relative quantification.

An essential prerequisite of this experiment is that the heavy proteins are fully labeled. As the cells grow in labeled medium and double, the original pool of unlabeled amino acids is diluted. To illustrate, consider a cell culture exposed to medium containing a stable isotope labeled amino acid at an RIA of 1.0. After one doubling time (D), the cell biomass would have increased by a factor of 2, so all proteins will be (at a minimum) 50% labeled. In practice, many proteins will be much more highly labeled than this because they have been turned over intracellularly during the first doubling time. In fact, the only proteins that attain the minimal labeling of 0.5 are those that are turned over at virtually imperceptible rates. By contrast, a protein with a half-life of 0.1 D would be virtually fully labeled even within that first doubling period; 50% of this protein is replaced within one-tenth of the cell doubling time. Of course, because it is impossible to know the turnover rate of each protein in advance, it is necessary to ensure that even slow turnover proteins are fully labeled. After 1 D, 50% of these slow turnover proteins would be labeled; at 2 D, 75%; 3 D, 87.5%; 4 D, 94%; 5 D, 97%; 6 D, 98%; and 7 D, >99%. Seven doubling times should ensure that all proteins are fully labeled. It is unfortunate that some studies do not provide this precise methodological information. Without an appreciation of the need for complete labeling, cells may be only partially labeled, which in subsequent comparative analysis will bias the expression ratios.

The majority of the studies described so far have determined the "relative" abundances of protein in two samples, one labeled with the heavy amino acid and the other unlabeled. For example, protein changes during muscle differentiation were monitored using [$^{2}H_{3}$]leucine as the labeled amino acid (17). Although quantification was consistent, analysis was hampered by the overlap between the isotopomers of heavy and light peaks, requiring a correction to peak intensities. Quantification was confirmed at the level of fragment ions during MS/MS. Because a goal for proteomics is full automation, more recent studies have employed stable isotope amino acids, providing a mass difference of 4–6 Da where overlap of isotopomers is minimized. For example, [$^{13}C_{6}$]lysine has been used to label *S. cerevisiae* proteins (35). Endopeptidase Lys-C generated peptides were analyzed by high resolution, single dimension reversed phase LC and FTICR-MS using automated data-dependent multiplexed MS-MS. Cleavage with endopeptidase Lys-C ensured that peptides (unless containing a missed cleavage or originally at the protein C terminus) would contain only one lysine and the use of ^{13}C-labeled lysine ensured reliable co-introduction of heavy and light variants into the FTICR-MS. Peptide pairs differing in mass by 6.02, 3.01, or 2 Da (singly, doubly, or triply charged ions, respectively) were selected for MS/MS, and identification of *y* ions in the spectrum was facilitated by the consistent mass offset, promoting efficient automated interpretation. However, sample complexity was high, and additional steps to reduce sample complexity would seem warranted for the analysis of more complex proteomes. AQ: C

Whereas the above study analyzed soluble, cytosolic proteins, other studies have attempted the quantification of all proteins, including membrane proteins (36). This necessitated solubilization in SDS and 1DGE separation. [$^{13}C_{6}$]Arginine was the chosen label, resulting in labeling of approximately half of the peptides after tryptic digestion. This approach worked well for quantification up to an 8-fold difference in abundance. Some label was found in proline; although not overly problematic, this was presumably a consequence of arginine catabolism.

Relative quantification using comparison of the signal intensity of heavy and light variants of the same peptide is reliable up to a dynamic range of 6–8, and the reproducibility of such measurements has allowed confidence in the significance of changes of greater than 20%. However, major goals for proteomics are full automation and the determination of the absolute quantity of each protein within a sample. Full automation is greatly facilitated by the avoidance of gel elec-

trophoresis and the absolute co-migration of heavy and light variants before introduction to the mass spectrometer. Absolute quantification via mass spectrometry would require the simultaneous analysis of a known quantity of individual peptides within a sample. Oda *et al.* (37) attempted absolute quantification of a single protein by mixing known quantities of unlabeled protein with labeled sample before analysis by 1DGE, tryptic digestion, and MALDI-ToF MS. Although this method used $[^{15}N]H_4Cl$ labeling, it is equally applicable to proteins labeled with amino acids. The method was successful, but the labor involved makes it only viable for a handful of proteins. Finally, we note with interest a recent study in which proteins in intact rats were labeled with stable isotopes by feeding a diet containing algal cells enriched with ^{15}N (38).

Use of Stable Isotope Amino Acids to Assist in Protein Identification

Although an increase in the accuracy of mass determination, as is provided by newer instruments (and in particular FTICR) will potentially reduce the number of data base matches from which a protein may be identified, the frequency of post-translational modifications limits its impact. However, protein identification can be significantly aided by partial amino acid composition data, and this can be readily obtained by comparing heavy and light variants of the same protein or peptide. Veenstra *et al.* (39) used incorporation of $[^2H_{10}]$leucine into *Escherichia coli* proteins and rapid separation by capillary isoelectric focusing. The masses of labeled and unlabeled proteins were compared using FTICR-MS, and the number of leucine residues was used to aid in identification. Heavy and light versions of the same protein were identical in their distribution of charge states and the mass difference between them, making calculation of their masses easy. They used these data to uniquely identify a number of proteins, although these authors did not address the potential loss of the α-carbon deuteron before incorporation. This general approach, although suitable for small genomes, is not sufficient for unique identification of proteins in complex genomes and has since been expanded by incorporating up to six different labeled amino acids (separately) to give information on amino acid composition and allow unique identification of peptides or intact proteins (28–32) without the need for tandem mass spectrometry to derive fragmentation series. The requirement to carry out multiple labeling experiments and multiple analyses detracts somewhat from the considerable gain achieved in protein identification. An additional problem is the need for several different labeled amino acids, ideally differing from the unlabeled version by a minimum of 4 mass units and the requirement for expensive ^{13}C-labeled versions to ensure co-elution when LC is used.

An alternative method for rapid protein identification has been largely directed at proteomes of lower complexity and minimal post-translational modification (19) based on the identification of a unique peptide for each protein, which when used with a mass spectrometer of high mass accuracy can be used as a unique identifier, a biomarker for that protein. For proteomes of organisms that will be subjected many times to different stimuli, the identification of an accurate mass tag for each protein will be worthwhile, allowing these masses to be selected and quantified and changes in protein abundance under different conditions be rapidly established without the redundancy involved in quantifying several peptides per protein and repeated identification. Although Smith *et al.* (19) used ^{15}N labeling and experienced the expected small discrepancies in co-elution of heavy and light versions of the same accurate mass tag (problems easily overcome by using ^{13}C-labeled amino acid precursors), they demonstrated that such an approach was feasible. For a small proteome, such as that of *Deinococcus radiodurans*, 51% of the tryptic peptides at 1 ppm measured mass accuracy would give a unique accurate mass tag. For application of this method to a genome as complex as the human genome, it is argued that a peptide fractionation procedure that yields 10 fractions followed by LC-FTICR might be sufficient. However, the impact of diverse post-translational modifications could be expected to lead to complications in interpretation.

Use of Stable Amino Acids to Measure the Rate of Protein Turnover

The current focus of many studies in proteomics is to determine the changes in levels of individual proteins; in this respect stable isotope labels can be of considerable value in comparative studies. However, a very different outcome of stable isotope labeling is obtained if the exposure to precursor is of such a short time that labeling is incomplete. Under these circumstances, the extent of labeling of peptides in single proteins is dictated by the growth rate of the system, and the rate of intracellular turnover of the protein. Short labeling windows can therefore access the turnover rates of individual proteins, and because these proteins can be subsequently isolated (whether by gel based methods, by LC separation of peptides, or by alternative approaches to proteome simplification), it is now feasible to measure the rate of turnover of individual proteins in the proteome. This information is going to become increasingly important as systems biology thinking starts to integrate transcriptome, proteome, and metabolome data. The often-reported imperfect correlation between transcriptome and proteome data may reflect the fact that the concentration of a protein in the cell is under the control of two processes, synthesis and degradation. For a constant ribosomal activity, an increase in mRNA concentration in the cell will enhance the rate of synthesis proportionately but will only result in an increase in protein levels if the rate of degradation remains unchanged, which is not always the case. For example, the subunits of some protein complexes are overproduced such that unassembled sub-

units are rapidly degraded; in such instances, the quantity of a protein in the cell may reflect the assembly of the complex rather than the quantity of the subunit that is synthesized. In addition, the literature is replete with examples of protein ligands, such as substrates, directly modulating the intracellular stability of a protein. Under these circumstances, the intracellular concentration of a protein can change without any change in the amount of the cognate mRNA. Such considerations are compelling reasons to express the concentration of any single protein in a proteome in terms of rates of synthesis and degradation. The rate of synthesis is directly linked to the transcriptome (via metabolic activity), and the rate of degradation is much more directly coupled to the metabolome and post-translational events such as supramolecular complex assembly.

The behavior of a protein pool can most simply be expressed as the rate of synthesis, the rate of degradation, and the protein pool size. Knowledge of any two of these parameters allows calculation of the third. It is often assumed that the calculation of protein pool size is the simplest of the three parameters to acquire, and this is usually measured. Thereafter, measurement of the rate of synthesis allows calculation of the rate of degradation or vice versa.

Synthesis measurements monitor the incorporation of label into protein; degradation experiments measure loss of label from protein. Although these two approaches might seem to have a formal equivalence, they bring with them unique problems. In biosynthesis studies using stable isotope tracers the extent of incorporation of the label might need to reach 10% of the protein, and even if the precursor RIA can be manipulated to be near unity this would still require, in the absence of growth, 15% of one half-life. For a global study, the protein with the lowest turnover would dictate this. In degradation studies, prelabeled protein is "chased" with unlabeled precursors. There are two difficulties with this approach. First, producing prelabeled material would require elongated exposure to the precursor pool, which might be difficult to achieve. Second, as prelabeled proteins are degraded, the labeled amino acids that are released become available for reincorporation into newly synthesized protein. This has the effect of artifactually elongating the measured half-life of the proteins. Nevertheless, we used stable isotope incorporation into *S. cerevisiae* growing in glucose-limited medium at steady state and measured the turnover of a number of abundant proteins. The label was [$^2H_{10}$]leucine, and *S. cerevisiae* were grown in a chemostat for more than seven generations to ensure full labeling. A large excess of unlabeled leucine was then added and samples taken at various stages during a 50-h chase. Proteins were separated by 2DGE and tryptic peptides analyzed by MALDI-TOF MS. Protein turnover followed first order kinetics and was remarkably consistent for multiple peptides from the same original protein. Moreover, sampling at a single time point generated comparable turnover rates (21).

When radioisotopes are used, complications are ameliorated by the ability to monitor tracer levels of labeling. The most common radiolabeling method for measurement of total protein synthesis is the "flooding dose" method, in which a large bolus of precursor is delivered in such a fashion as to swamp the endogenous pools. Hence, the precursor specific radioactivity can be taken to be that of the flooding dose material. This is of course unfeasible in stable isotope methods for turnover measurement in that mass spectrometers routinely used in proteomics cannot measure low levels of incorporation.

In experiments in which it is not possible to attain fully labeled precursor pools, precise determination of rates of protein synthesis requires knowledge of the value of precursor RIA. This can be accessed by recovery of the precursor from the tissue sample, but there is some disagreement as to whether the amino acid pool or the aminoacyl tRNA is the most appropriate reporter of the RIA of the immediate precursor of protein synthesis. However, under circumstances in which the precursor RIA is less than unity, direct analysis of peptide mass isotopomers can yield the RIA of the immediate precursor of the protein irrespective of the chemical nature or specific pool location (34, 40). This is a major advantage of stable isotope labeling. We have recently demonstrated that mass isotopomer distribution analysis can be used to derive the RIA of a precursor pool when a stable isotope amino acid is administered in the diet to an intact animal (24).

Use of Stable Isotope Labeling to Monitor Protein Modification

To follow changes in the modification of proteins, there is a generic problem caused by the alteration in separation properties of the modified variant. Apart from the obvious change in mass, the difference in other properties (charge, hydrophobicity, and shape) results in failure to co-migrate/co-elute when separated by 2DGE, capillary isoelectric focusing, HPLC, or ion exchange chromatography. 1DGE separations (with the exception of modification by glycosylation) are less affected by these modifications. For reliable quantification by mass spectrometry, unmodified and modified variants (protein or peptides) would not only need to be analyzed in the same spectrum but must also have the same ionization efficiency. Because the latter cannot be guaranteed, reliable quantification of modified relative to unmodified protein is not possible; instead, studies have focused on monitoring changes in the amount of the modified variant between two or more different states.

So far, most global phosphoproteome studies have not used stable isotope metabolic labeling. Instead, methods have been developed that result in the specific isolation of proteins or peptides that have been modified by phosphorylation, thereby reducing sample complexity. These include two methods that can be applied directly to protein mixtures (specific immunoprecipitation using antibodies specific for

AQ: D

phospho-tyrosine, -threonine, or -serine and immobilized metal (Fe^{3+}) affinity chromatography) and three methods that involve chemical modification of phosphate groups (phosphoramidate chemistry (41), β-elimination-Michael addition (37), and phosphoprotein ICAT (42)), which then facilitate affinity selection. These methods, together with MS/MS analysis, have all been used with reasonable success to identify phosphorylated proteins and the sites of phosphorylation. For comparative phosphoproteomics, stable isotopes have been incorporated during chemical modification methods (37, 42). With all these studies, additional work is required to distinguish between an increase in the proportion of a given protein that is phosphorylated and an overall increase in the abundance of a protein with a fixed proportion carrying the modification.

A handful of studies have used metabolic labeling to monitor protein modifications and phosphorylation in particular. Oda *et al.* (37) observed the differences in abundance of peptides derived from two *S. cerevisiae* strains differing only in their ability to express G_1 cyclin. A 50/50 mixture of the two strains (one labeled in ^{15}N medium) was analyzed, and the parent proteins of tryptic peptides showing unequal abundance were identified. A study of other peptides from the same parent proteins revealed those peptides that differed as a result of post-translational modification rather than a change in the overall abundance of the protein in the two strains. The type of modification was then established during MS/MS. In another study, [$^2H_3$]serine labeling coupled with 1DGE separation was used to allow immediate focus on peptides that could serve as substrates for protein kinase A (32). The phosphorylation of a histone protein from human skin fibroblasts in response to low dose irradiation was demonstrated.

A number of studies have assessed the phosphorylation of individual proteins. This has been achieved by generating a variant of the protein such that it is readily purified from whole cells (for example, by including a His tag or an immunoprecipitable epitope in the gene). These constructs were then transiently expressed in cells grown in labeled or unlabeled medium, and the labeled cells were subjected to various stimuli. Labeled and unlabeled cells were then mixed, the protein under study recovered, and differences in modifications established by mass spectrometry (43). The study can be focused toward the modification of specific residues, for example tyrosine, by using tyrosine as the labeled amino acid and selecting peptides that differ in intensity in mass spectrometry for further study by MS/MS (44).

Bioinformatics for Stable Isotope Labeling

Several types of experiments are based on the incorporation of stable isotope-labeled amino acids, and for each application a different approach to data analysis is required. Perhaps most simply, MALDI-TOF spectra comprising a mixture of peptides labeled with heavy and light variants of an amino acid can be inspected manually, and the relative intensities of the two ions can be assessed. This assumes that each ion has an identical response factor in the instrument, which is a relatively safe assumption. There may be added complications when product ion spectra are used for quantification (45), but a recent study has confirmed a satisfactory correlation between difference gel electrophoresis and metabolic stable isotope labeling ($r^2 = 0.89$) (46).

Few tools for automated identification and quantification of isotopically differentiated peptides are generally available. Two tools, however, are worthy of note. The first, MS-Isotope, is part of the Protein Prospector package (Ref. 47; prospector.ucsf.edu) and is a web-based tool that allows the natural isotope distribution profile of any peptide to be calculated. This facilitates calculation of relative abundances of peptides when, in particular, the natural isotope profiles of the unlabeled and labeled peptides overlap. The second tool, MS-Quant (Ref. 48, msquant.sourceforge.net) is a stand alone application that is able to extract quantitative data from isotope-labeled mass spectra.

A simple approach to identification of isotopically labeled pairs of peptides is to code them in search algorithms as a pseudo-post-translational modification. Search algorithms will then identify stable isotope-labeled amino acids as mass-modified peptides and are of course tolerant to multiple instances of the same amino acid in a peptide. This can also be applied to analysis of MS/MS spectra.

On the other hand, if a peptide pair is readily identifiable by manual inspection of the mass spectrum, it is possible to include the additional information about the amino acid composition in the search term. The MASCOT (Ref. 49, www.matrixscience.co.uk) search engine in particular includes the capability to encode amino acid composition data using appropriate search terms. Thus, a search string of the form "[M+H]+ comp(Leu[n])" not only submits the peptide mass to the search algorithm but also restricts the search space further by including the number of leucine residues (n) present in the peptide.

However, a mass spectrum containing both labeled and unlabeled variants of a number of peptides is complex, particularly when the precursor RIA is less than unity. In our experience, there is no automatic way to process such complex spectra, and it is common to resort to manual interpretation and analysis. The lack of automatic processing of spectra, made more complex by the use of stable isotope labeled precursors, is a disincentive to wider adoption of this approach.

Conclusions

Although there has been considerable interest in stable isotope labeling *in vitro*, the scope and utility of labeling *in vivo* is only now being explored in detail. Particularly suited to cells grown in culture, there is an emerging experience with more

complex systems such as intact animals. These studies have confirmed that for comparative proteomics, metabolic labeling may not be an option in complex systems in which complete labeling is difficult if not impossible to achieve. However, the ability to monitor rates of labeling, even in such systems, allows one to explore the dynamics of the proteome.

With the emergence of systems biology, it is increasingly clear that proteomics must undergo a paradigm shift and evolve from relative quantification to absolute quantification. Stable isotope-labeled internal standards will become increasingly important in absolute quantification, although this is beyond the scope of this review. In addition, there is an increasing need to understand variation in protein expression in terms of protein turnover, which requires robust methods to measure dynamics on a proteome wide scale; the methods that are being developed are largely based on stable isotope labeling strategies. Many more developments in the use of such strategies in complete and definitive proteome comparison may be anticipated.

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